Evaluation of oxidative stress induced by insecticides on *Brassica oleracea* infested with *Spodoptera litura*

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Abstract: Cauliflower is an important vegetable crop grown in Pakistan which is damaged by many insect/pests including *Spodoptera litura* (army worm). These insect/pests feed on leaves and deteriorating the quality and quantity of cauliflower. Different insecticides are used for the control of this pest and causing a certain disturbance in biochemical constituents of the plant. The present project was designed to study the lethal effects of different insecticides (Bifenthrin, Emamectin Benzoate and Lufenuron) on army worm and physiological effects on two different Cauliflower cultivars (Shehzadi and Snow Crown) in the form of oxidative stress. The experiment was conducted in Research Area, Department of Entomology, University of Agriculture, Faisalabad (Pakistan). The experiment had three treatments (Bifenthrin, Emamectin Benzoate and Lufenuron) with one control and had laid out in tri-replicated Randomized Complete Block Design (RCBD). Insecticides were applied through foliar application method with Knap sack sprayer. Pest scouting was done to check mortality rate of S. litura under different treatments (Bifenthrin, Emamectin Benzoate and Lufenuron). A laboratory experiment was also conducted to check the mean percent mortality of *Spodoptera litura*. In pots, Emamectin Benzoate showed maximum mortality (96.66%) after 7 days of insecticide application. Bifenthrin showed minimum mortality (64.07%) after 7 days of application. Under field conditions reduction in infestation was 94% by Emamectin Benzoate. Leaf samples were collected before and after the insecticides application to check the nature and severity of oxidative stress. CAT activity was reduced after insecticide application as compared to control treatment. SDO, POD, MDA and proline activity was increased after insecticide application. Salicylic acid was applied through foliar to overcome the stress effects produced by insecticides. It was proved that Salicylic acid enhances the CAT activity but Salicylic acid reduces SOD, POD, MDA and proline activity.

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1. Introduction:

Pakistan is fundamentally an agricultural country and Pakistan's economy depends on agriculture. Gross domestic product is supported by farming is almost 24% and the aggregate work constrain that is straightforwardly required with horticulture is around 44%. Nearly, all type of crops is grown in plains of Indus River (Anonymous, 2014).

The number of small farms is increasing in Pakistan due to division, sale, and resale of agricultural land over times. The cultivation of cash crops on small farms is not beneficial due to the long duration and unavailability of modern technology. Growing of short duration crops and vegetables instead of traditional long duration farming patron is more beneficial (Bakhsh *et. al*., 2004).

In Pakistan, production of vegetables is limited near to cities and large towns and cropping area under vegetables is only 2% of the total cultivated area (Annonymous, 2002). Cauliflower (*Brassica oleracea* L.) is one of the major vegetables grown in the south and South East Asia (Maqsood *et al.,* 2016). Cauliflower has good nutritional value (Ahuja *et al.,* 2015). Pakistan is in top ten cauliflower producing countries. The area under cultivation of cauliflower in Pakistan is 11400 ha and yield is 183,333 hectogram/ha. Selection of good variety and production technology are two major factors that affect the profitability of crop (Siddiqui *et al.,* 2009).

Cauliflower is attacked by many insect pests and diseases. There are eight insect species and four diseases that limit the cauliflower production. (Ahuja *et al.,* 2012, 2013).

Army worm (*Spodoptera litura*) has a place in order Lepidoptera and family Noctuidae. *S. litura* feeds on leaves of more than 100 plants species. As per host plant study in 3 different territories in the cotton belt of Pakistan, uncovered twenty-seven distinctive plant species as host of *S. litura* which has

a place with 25 genera of 14 families (Ahmad *et al*., 2013).

Armyworm (*Spodoptera litura*) is one of the most destructive insects that attack cauliflower and causing 31% to 100% loss in yield and quality. It eats gregariously on leaves of crop and only mid rib is left on a leaf (Zhou *et. al.,* 2012). Armyworm has 5-6 overlapping generations in a year (Kaur *et. al., 2011*).

Severe infestation of armyworm leads towards the control through insecticides. Many insecticides are being used against armyworm includes conventional (OP's, Pyrethroids, permethrin, spinosad, emamectin benzoate and new chemistry insecticides (Khan *et al.,* 2011 and Carasi *et. al.,* 2014). Avermectins is a new group of insecticides that is eco-friendly and has promising results against armyworm (Bengochealt *et. al.,* 2014).

Intensive use of insecticides leads towards the production of chemicals, like lipid peroxidation (LOP) and reactive oxygen species (ROS) within the plant that damage plant tissues. High contents of these chemicals in plant cells lead towards cell death and disturbs normal functions in plants. (Hazarika *et. al.,* 2003).

Reactive oxygen species (ROS) production is deliberated as a basic result under many stress circumstances (Noctor and Foyer 1998). The production of ROS relies on intensity of the stress as well as on physico-chemical state in the cell. The enzyme inactivation, damage to DNA as well as lipid per-oxidation are outcomes of reactive oxygen formed within the cell (Shewfelt and Purvis 1995).

ROS affects the cell through the combination of following factors: the amount of ROS produced as well as bio-chemical prominence of cell. But how and where ROS play role in cell death program (transduction cascade) are still to be find out (Semenza 1999).

Over production of ROS is a quick response of plants to environmental conditions. TBARS contents are accumulated in cells when lipid peroxidation occurs. Lipid peroxidation is the first step in the process of membrane damage by insecticides (Parween *et al.,* 2012).

Proline also accumulates in plants when they are in stress like drought, salinity and any other environmental factor. Detoxification of these ROS, protection of membrane from degradation and stabilization of proteins are the functions of proline in the cell (Parween *et. al.,* 2012).

To overcome the stress, plants produce certain chemical. There are some oxidants (catalase (CAT), super oxide dismutase (SOD), peroxidase (POD), ascorbate peroxidases (APX), glutathione reductase (GR)) and antioxidants (ascorbate content and glutathione content) in plants to convert these ROS into less toxic substances in nature (Inze *et. al.,* 1995).

ROS produce free oxygen radicals that are toxic to plants. SOD converts free oxygen radicals to hydrogen peroxide (H_2O_2). H_2O_2 is less toxic than free oxygen radicals. The first stable compound, H_2O_2, is amongst ROS synthesized within plant cell during the normal physical situation and during stress conditions. Therefore, H_2O_2 is most likely compound for ROS-mediated signal transduction. H_2O_2 is the stable compound as well as able to permeate cell membrane as an uncharged molecule and can be relocated to action site (Foyer *et al*., 1997).

Aerobic organisms represent SOD presence in all subcellular parts that are susceptible to oxidative stress (Bowler *et al*., 1992). SOD higher level activities are witnessed to ROS production in plant cells. More SOD activity is reported in crops on which insecticides or herbicides are applied (Parween *et al.,* 2012).

POD breakdowns the H_2O_2 and lignin biosynthesis. APX is one type of POD that uses ascorbate to remove H_2O_2. In plants, SOD and APX activities must be in balance, so that H_2O_2 produced in SOD activity is removed by APX activity. CAT also has the similar type of action as it removes H_2O_2 from plants (Parween *et al.,* 2012).

SA (Salicylic Acid) is signaling molecule that induces tolerance in plants from abiotic stress. It increases the dry matter yield in all type of soil conditions. If it is applied externally, it improves the membrane deterioration significantly. SA application decreases lipid peroxidation rate in plants but the concentration of H_2O_2 increase significantly. If applied in appropriate concentration salicylic acid boosts the antioxidant activity in plants (Hayat *et al.,* 2010).

2. Materials and methods:

The present research work was designed to study comparative effects of different insecticides (Lufenuron, Bifenthrin and Emamectin benzoate) producing oxidative stress in Cauliflower which was used against *Spodoptera litura*. The experiment was conducted at Research Area, Department of Entomology, University of Agriculture, Faisalabad (Pakistan).

i. Transplant of Nursery

One-month-old plants of cauliflower were transplanted in the field on ridges in last week of October. Two cultivars Shahzadi and Snow Crown were sown. Each experiment was repeated 3 times.

ii. Pest Scouting and Insecticide Application

Pest scouting was done every week after nursery transplantation. When the population of Spodoptera litura appears insecticides were applied. Three insecticides Bifenthrin, Emamectin benzoate and Lufenuron @ 250 ml, 200 ml, and 200 ml were

applied respectively.

Experiment # 01:

Efficacy of insecticides was checked against army worm on cauliflower plants sown in laboratory condition. Cauliflower plants were sown in pots and covered with net cloth. Selected number of army worm larvae was released on each plant. Insecticides were applied to plants according to CRD and plants were again covered with net cloth. Mortality of army worm was recorded after four and seven days of insecticide application. Each experiment was repeated three times.

Reduction in infestation of army worm was recorded from the field experiment. Average larval population on cauliflower plants was recorded and insecticides were applied. Then the number of larvae per plant was recorded from each treatment after week 1, week 2 and week 3 of insecticide application. Then the reduction in infestation was measured by comparing the average number of larvae before insecticide application and the average number of larvae after insecticide application.

Experiment # 02

Sample Collection and Stress Analysis

Fresh leaf was collected after 7, 14 and 21 days of insecticide application. The chemical analysis was performed to get the concentrations of lipid peroxidation rate, superoxide dismutase, peroxidases, catalases, and proline.

A. Catalase (CAT) action

Catalase activity was measured by the technique of Liu *et al.,* (2000) with few changes. Chemicals required for measurement of CAT activity were 50 m*M* phosphate buffer with pH 7, 0.1 mL enzyme extract and 5.9 m*M* H_2O_2. As soon enzyme extract was added reaction starts and change in absorbance of the reaction solution was observed at 240 nm.

B. Superoxide dismutase (SOD) action

The action of SOD was determined by measuring its capacity to restrain the photoreduction of nitro blue tetrazolium (NBT) taking after the method for Giannopolitis and Ries (1977). The response arrangement contained 0.222 gm methionine in 15 mL of distill water, 0.015 gm of NBT in 17.5 mL of distill water, 0.0375 mL of Triton-X in 17.5 mL of distill water, 0.0132 gm of Riboflavin in 17.5 mL of distill Water and 0.2 M cushion.

Assay	Volume
Phosphate buffer	500 µL
Methionine	200 µL
NBT	100 µL
Triton-X	200 µL
Riboflavin	100 µL
Sample	100 µL
Dist. water	800 µL

The reaction solution was placed under UV lamp in the test tube for 15 minutes then add Riboflavin. Light absorbance was measured at 560 nm with spectrophotometer.

C. Peroxidase (POD) action

Chemicals required for POD activity were 50 m*M* phosphate buffer with pH 5, 20 m*M* guaiacol, 40 mM H_2O_2, and 0.1 mL enzyme extract. Change in absorbance is counted at 470nm.

The activity of each enzyme was expressed on the protein basis. The protein concentration of the crude extract was measured by the method of Bradford (1976).

D. Estimation of MDA (lipid peroxidation) rate

The levels of malondialdehyde (unit/ml) were measured by using Thiobarbituric acid reactive substances.

Chemicals required

- Sodium dodecyl sulfate
- Thiobarbituric acid
- Acetic acid
- n- butanol
- Distilled water

Serum sample preparation

10% (w/v) sample was prepared using 10 mM buffer then centrifuge solution for 10 minutes at 13000 rpm and 4℃.

Protocol

The Thiobarbituric acid receptive substance in serum was assessed by the technique for Ohkawa et al (1979). In test tube 200 µl of serum test, 200 µl of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 0.8% TBA, 1.5 ml of 20% acidic corrosive arrangement (pH 3.5) and 4.0 ml distilled water was added. And after that was warmed in water at 90℃ for an hour.

E. Proline determination:

Proline from the dry leaf sample was evaluated by the strategy for the Bates *et. al.,* (1973). A specimen of 0.5 g crisp leaf tissue was homogenized in 10 ml of 3 % sulfosalicylic corrosive and the homogenate was separated through Whatman No. 2 channel paper. At that point 2.0 ml of the filtrate was blended with 2.0 ml corrosive ninhydrin arrangement (Ninhydrin (1.25 g) was broken up in 20 ml of frosty acidic corrosive and 20 ml of 6 Morthophosphoric corrosive thereafter put away and cooled at 4 °C) and 2 ml of chilly acidic corrosive in a test tube. This blend was brooded at 100℃ for an hour and after that cooled in an ice shower. At last, 4.0 ml of toluene were added to the arrangement and blended vivaciously by passing a ceaseless stream of air for 1-2 min. The chromophore containing toluene was suctioned from the fluid stage, warmed at room temperature and the absorbance was perused at 520

nm utilizing spectrophotometer (HITACHI, U2800) and toluene was taken as clear.

3. Results and discussion:

Armyworm is most destructive insect pest that attacks cauliflower causing 31% to 100% loss in yield and quality (Zhou et al., 2102) and controlled through different insecticides (Khan et al., 2011). Intensive use of insecticides leads towards the production of chemicals, like lipid peroxidation (LPO) and reactive oxygen species (ROS). High contents of these chemicals in plant cells lead towards cell death (Hazarika et. al., 2003). The production of reactive oxygen species (ROS) is reflected to be a basic result under a many of stress situations (Noctor and Foyer 1998). To overcome the stress, plants produce certain chemical. There are some Oxidants (catalase (CAT), super oxide dismutase (SOD), peroxidase (POD), ascorbate peroxidases (APX), glutathione reductase (GR)) and Antioxidants (ascorbate content and glutathione content) in plants to convert these ROS into less toxic substances in nature (Inze et. al., 1995).

Experiment 1: Table 1 showed the %age mortality of Spodoptera litura by different insecticides on cauliflower crop sown in pods. All insecticides showed increased mortality after 7 Days of insecticide application than 4 Days of application. After 4 Days of insecticide, application Emamectin showed maximum 82.222 % mortality followed by Lufenuron 64.074 % and least mortality was showed by Bifenthrin 60.74. After 7 days of application, Emamectin showed maximum 96.667 % mortality followed by Lufenuron 78.518 % and least mortality was showed by Bifenthrin 64.074.

Table 2 showed the %age reduction in infestation of Spodoptera litura by different insecticides on cauliflower crop sown in the field. All insecticides showed maximum mortality after the first week of insecticide application. Emamectin showed maximum mortality i.e. 94.97% followed by Lufenuron 81.74% and Bifenthrin 69.53%. After the 2nd week of insecticide application toxicity was decreased and Emamectin showed maximum 86.21 % mortality followed by Lufenuron 80.25 % and least mortality was showed by Bifenthrin 60.75. After the 3rd week of insecticide, application of Emamectin showed 77.33% mortality followed by Lufenuron 65.29% and least mortality was showed by Bifenthrin 51.79%.

Table 1: Average % Mortality of *Spodoptera litura* F. induced by different insecticides in cauliflower crop sown in pots

Treatment	Average %age Mortality	
	Day 4	Day 7
Bifenthrin	60.74074d	64.07407cd
Emamectin	82.22222ab	96.66667a
Lufenuron	64.07407cd	78.51852bc

Table 2: %age Reduction in Infestation of *Spodoptera litura* F. induced by different insecticides in cauliflower crop sown in field

Treatment	Average %age Reduction in Infestation		
	Week 1	Week 2	Week 3
Bifenthrin	69.539d	60.75e	51.79f
Emamectin	94.974a	86.219b	77.33c
Lufenuron	81.747bc	80.258b	65.296de
Control	-7.787g	-22.352h	-54.397i

Experiment 2: Table 3 showed that Maximum CAT activity was in control treatments 92.806 IU/mg of protein and decreased significantly to 85.54 IU/mg of protein after the application of insecticide. Decreased CAT activity was reported due to insecticide use (Bashir et. al., 2007) and by herbicides in the wheat crop (Jaleel et. al., 2009). Temperature also decreases the CAT activity in Haseolus vulgaris L. (Nagesh and Devaraj 2008). Rastgool and Alemzadeh (2011) also reported the decrease in CAT activity under Pb stress. The decreased in CAT activity was due to increase in lipid per oxidation rate increases under insecticide stress. And increased lipid per oxidation rate reduces the H2O2 (Halliwell and Gutteridge 1985).

Table 4 showed that variation in SOD activity. SOD is a vital part of a plant's anti-oxidative defense mechanism. SOD converts free oxygen radicals to H2O2 i.e. less toxic to plants than free oxygen radicals (Haddad et. al., 2009). SOD activity was increased in insecticide treated plants as compared to control. SOD activity in control was 18.69 IU/mg of protein and plants treated with Emamectin showed 64.64 IU/mg of protein. SOD activity was more during the first week of insecticide application as compare to 3rd week of insecticide application. Similar results were found by Haddad et. al., (2009) because aged leaves had lower antioxidant concentrations than young leaves.

Table 5 showed variation in POD activity. POD is a 3rd important enzyme in plant anti-oxidant defense system. POD break down H2O2 and converts

it to lignin (Bowler et. al., 1992). POD activity must be in balance to SOD activity so that H2O2 produced by SOD must be eliminated by POD for proper chloroplast function (Asada 2006). POD activity was significantly low in the control treatment (28.985) as compared to plants under insecticide exposure (36.573). A similar type of results was reported under fungicide (Gopi et. al., 2007) and herbicide (Jianga et. al., 2010) application.

Table 6 shows the change in MDA concentration in the plant after insecticides application. The significant change in MDA concentration was observed after application of insecticides. MDA concentration increased significantly by all three insecticides (33.43 µg/ml) as compared to control (16.51 µg/ml). The similar type of results was found by Parween et. al., (2012) on Vigna radiate in response to chlorpyrifos. The increase in MDA concentration shows that ROS production is one of the major toxic effects of insecticides. Bashir et. al., (2007); Song et. al., (2007) described the effects of deltamethrin on *Glycine max* L. leaf and roots respectively, and found similar results.

Table 7 shows the change in Proline concentration in the plant after insecticides application. Higher concentration of Proline accumulates in plants under stress of insecticides, drought, salinity and other environmental stimuli (Ashraf and Foolad 2007). Proline detoxifies ROS and stabilizes enzyme tolerance to stress (Mittler 2002). Proline concentration increased significantly in plants under insecticide treatment (14.895 µg/ml) as compared to control (6.234 µg/ml). Similar results were observed by Parween et. al., (2012) on Vigna radiate in response to chlorpyrifos and by Wu et. al., (2010) on rice in response to insecticides.

Table 3: Mean for Variation in CAT action (IU/mg) for 3 weeks of insecticide application on *Brassica oleracea* L.

Insecticides	Week 1		Week 2		Week 3	
	Shehzadi (V1)	Snow Crown (V2)	Shehzadi (V1)	Snow Crown (V2)	Shehzadi (V1)	Snow Crown (V2)
Bifenthrin	89.564±0.388cd	89.068± 0.629cd	86.28± 0.193hi	87.336± 0.360efg	86.469± 0.409gh	86.20± 0.111ij
Emamectin	88.49±0.122def	91.170± 0.275bc	87.11± 0.445ef	86.789± 0.249fgh	86.007± 0.238ij	88.63± 0.434d
Lufenuron	87.983±0.555de	88.336± 0.338de	85.31± 0.360j	85.763± 0.292j	85.460± 0.511j	87.99± 0.307d
Control	92.079±0.467ab	92.979± 0.334ab	93.32± 0.584a	92.289± 0.333ab	93.104± 0.369ab	92.449± 0.190ab

Table 4: Mean for Variation in SOD action (IU/mg) after 3 weeks of insecticide application on *Brassica oleracea* L.

Insecticides	Week 1		Week 2		Week 3	
	Shehzadi (V1)	Snow Crown (V2)	Shehzadi (V1)	Snow Crown (V2)	Shehzadi (V1)	Snow Crown (V2)
Bifenthrin	37.84± 0.809cd	58.37± 2.12b	20.03± 1.27h	30.40± 1.86defg	21.14± 0.838gh	33.71± 1.00de
Emamectin	64.64± 1.39ab	69.15± 2.46a	45.31± 0.297c	26.00± 0.370efg	39.09± 0.811cd	24.98± 0.77efgh
Lufenuron	32.40± 1.12def	61.73± 2.53ab	23.36± 1.10fgh	33.97± 1.34de	29.73± 0.83def	22.85± 1.34gh
Control	23.79± 1.01fgh	37.28± 1.18cd	20.05± 1.63h	23.09± 4.99fgh	18.69± 0.603h	21.57± 2.19gh

Table 5.4b: Mean for Variation in POD action (IU/mg) after 3 weeks of insecticide application on *Brassica oleracea* L.

Insecticides	Week 1		Week 2		Week 3	
	Shehzadi (V1)	Snow Crown (V2)	Shehzadi (V1)	Snow Crown (V2)	Shehzadi (V1)	Snow Crown (V2)
Bifenthrin	32.806± 0.123a	34.166± 0.169efg	34.109± 0.210ef	36.150± 0.339cd	35.385± 0.179def	37.761± 0.414bc
Emamectin	33.066± 0.160ghi	30.228± 0.185l	34.544± 0.270de	35.086± 0.302de	34.663± 0.0927def	35.622± 0.136de
Lufenuron	34.030± 0.183efg	35.499± 0.297de	32.160± 0.256jk	40.172± 0.469a	37.968± 0.296b	33.638± 0.193fgh
Control	31.720± 0.242kl	26.250± 0.249m	31.821± 0.233kl	27.619± 0.696m	32.468± 0.255ijk	27.029± 0.743m

Table 6: Mean for Variation in MDA action (µg/ml) after 3 weeks of insecticide application on *Brassica oleracea* L.

Insecticides	Week 1		Week 2		Week 3	
	Shehzadi (V1)	Snow Crown (V2)	Shehzadi (V1)	Snow Crown (V2)	Shehzadi (V1)	Snow Crown (V2)
Bifenthrin	29.119±0.453de	37.752± 0.594a	29.083± 0.424de	32.032± 0.755bcd	20.653± 0.441gh	35.749± 0.916ab
Emamectin	31.277±0.478cd	33.963± 0.563ab	30.054± 0.599cd	21.32± 1.31g	19.035± 0.322ghi	26.876± 0.934f
Lufenuron	27.992±0.344ef	28.040± 1.29ef	27.668± 0.323ef	17.50± 1.44ghi	35.713± 0.428ab	18.16± 1.15ghi
Control	17.584±0.963gh	16.253± 0.146i	16.577± 0.509i	16.397± 0.187i	16.757± 0.161hi	16.265± 0.213i

Table 7.4b: Mean for Variation in Proline action (µg/ml) after 3 weeks of insecticide application on *Brassica oleracea* L.

Insecticides	Week 1		Week 2		Week 3	
	Shehzadi (V1)	Snow Crown (V2)	Shehzadi (V1)	Snow Crown (V2)	Shehzadi (V1)	Snow Crown (V2)
Bifenthrin	15.550±0.671bc	13.726±0.542cd	15.835± 0.593bc	9.443± 0.422fg	3.375± 0.169j	8.076± 0.203fgh
Emamectin	12.843±0.511d	12.511±0.573de	16.348± 0.247b	13.442± 0.668cd	3.242± 0.164j	8.133± 0.543fgh
Lufenuron	9.187± 0.372fg	9.538± 0.143fg	9.909± 0.242fg	10.336± 0.379ef	7.526± 0.508gh	12.710± 0.404de
Control	6.898± 0.437hi	7.658a±0.321gh	9.918± 0.758fg	6.908± 0.445hi	6.025± 0.171i	6.443± 0.293i

4. Conclusion:

The current experiment evaluates the responses of *Brassica oleracea* L. against three different insecticides including Bifenthrin, Emamectin, and Lufenuron also elucidated the capability of pesticide metabolizing anti-oxidative enzyme system. Triggering of metabolic processes in plant cells in response to chemical stress is demonstrated in (a) accretions of proline, and (b) up rise in numerous enzymatic and non-enzymatic antioxidants in many plant parts, thus signifying that effectiveness of Asc–Glu cycle increases as to detoxify ROS in cells. Likewise, it exposed the convoluted indication about the breakdown of insecticides molecules by the greater action position of oxidoreductase enzymes. Such biochemical outcomes can be understood as internal tolerance mechanisms and may permit us to make improved approaches for decreasing the risks of insecticide adulteration in crop production. Enzyme expression at the gene level and their breakdown studies by identifying intermediate degradation compounds are the problems for future concerns.

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